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Heterotypic RPE-choroidal endothelial cell contact increases choroidal endothelial cell transmigration via PI 3-kinase and Rac1

Lynda J. Peterson[a,†], **Erika S. Wittchen**[b,†], **Pete Geisen**[a], **Keith Burridge**[b], and **M. Elizabeth Hartnett**[a§]

a*Department of Ophthalmology, 6135 Neuroscience Research Building, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7041*

b*Department of Cell and Developmental Biology and Lineberger Comprehensive Cancer Center, 12-026 Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295*

Abstract

Age-related macular degeneration (AMD) is the major cause of non-preventable blindness. Severe forms of AMD involve breaching of the retinal pigment epithelial (RPE) barrier by underlying choroidal endothelial cells (CECs), followed by migration into, and subsequent neovascularization of the neurosensory retina. However, little is known about the interactions between RPE and CECs and the signaling events leading to CEC transmigration. While soluble chemotactic factors secreted from RPE can contribute to inappropriate CEC transmigration, other unidentified stimuli may play an additional role. Using a coculture model that maintains the natural structural orientation of CECs to the basal aspect of RPE, we show that “contact” with RPE and/or RPE extracellular matrix increases CEC transmigration of the RPE barrier. From a biochemical standpoint, contact between CECs and RPE results in an increase in the activity of the GTPase Rac1 within the CECs; this increase is dependent on upstream activation of PI 3-K and Akt1. To confirm a link between these signaling molecules and increased CEC transmigration, we performed transmigration assays while inhibiting both PI 3-K and Rac1 activity, and observed that both decreased CEC transmigration. We hypothesize that contact between CECs and RPE stimulates a signaling pathway involving PI 3-K, Akt1, and Rac1 that facilitates CEC transmigration across the RPE barrier, an important step in the development of neovascular AMD.

1. Introduction

Choroidal neovascularization (CNV) in the neurosensory retina is a major reason for rapid vision decline in age-related macular degeneration (AMD), the leading cause world-wide of non-preventable blindness (Resnikoff et al., 2004; Kulkarni and Kupperman, 2005; Chopdar et al., 2003). From clinical and pathological studies it is known that vision loss from CNV can occur after choroidal endothelial cells (CECs) are activated and migrate through a semi-permeable collagen and elastin structure called Bruch’s membrane, to make ‘contact’ with the retinal pigment epithelium (RPE), or its extracellular matrix (ECM), and then transmigrate the

§**Corresponding Author:** M. Elizabeth Hartnett, Email address: hartnet@med.unc.edu, Department of Ophthalmology, University of North Carolina Chapel Hill, 103, Mason Farm Road, CB#7041, 6135 NSRB, Chapel Hill, NC 27599, USA, phone (919) 966-1144; fax (919) 843-0749.
†Authors contributed equally.

RPE into the neurosensory retina (Stevens et al., 1997). However, little is known about the molecular interactions between CECs and RPE or its ECM, with respect to the signaling events triggered by their contact prior to CEC transmigration across the RPE. Understanding the signaling events that precede neurosensory retinal CNV is important to develop effective interventions to prevent vision loss due to neovascular AMD.

Cell migration is a multi-step process that involves extension of lamellipodia and filopodial protrusions at the leading edge of the migrating cell, formation of nascent adhesions within the leading edge, and the retraction and release of adhesions at the rear of the cell (Lauffenburger and Horwitz, 1996; Sheetz et al., 1999). This process involves dynamic rearrangements of the actin cytoskeleton and cyclic assembly/disassembly of multi-molecular adhesion complexes ultimately regulated by the activity of the small GTPases Rac1 and RhoA (Burridge and Wennerberg, 2004). Rac1 is the key regulator of actin polymerization and reorganization in cell-membrane protrusions during directed cell migration (Nobes and Hall, 1995). Rac1 activity has been measured in isolated pseudopodia of migrating cells (Cho and Klemke, 2002), or by FRET analysis of neutrophils (Gardiner et al., 2002) during directed migration, and has been shown to be increased at the leading edge where it is necessary for migration.

Rac1 has been shown to both activate and be activated by PI 3-K (Kotani et al., 1994; Posern et al., 2000). It has also been shown to be regulated through growth factor-receptor activation. We previously demonstrated that CECs extend cell membrane protrusions and transmigrate across a monolayer of RPE in response to a gradient of soluble VEGF produced by the RPE, and that neutralizing antibodies to VEGF only partially inhibited this transmigration, indicating other factors are important in CEC migration (Geisen et al., 2006a). In this study, we wished to determine whether contact of CECs with RPE was important for CEC transmigration, and if so, whether it resulted in activation of signaling pathways within the CECs to enable CEC transmigration. We postulated that the small GTPase Rac1 and proteins which regulate its activity might be key determinants in CEC transmigration.

2. Materials and Methods

2.1 Cell Culture

Primary human choroidal ECs (CECs) were isolated from donor eyes obtained from the North Carolina Eye Bank described in detail previously (Geisen et al., 2006a). ARPE 19 cells (RPE) were obtained from ATCC (Rockville, MD) and grown in DMEM/F-12 plus 10% FBS, and used from passage 15–20. CECs were grown in endothelial growth media (EGM-2) supplemented with 10% FBS and used from passage 2–4.

2.2 Transmigration Assay

Transmigration assays were performed with 8.0 μm pore size Transwells (Costar/Corning, NY) that permit cell transmigration across the filter. RPE (1.8×10^5) were grown for 72 hr on the underside of the Transwell inserts. Monolayer organization and ZO-1 localization to cell-cell junctions was used as an indicator that RPE grown under these conditions exhibit polarized features (Supplemental Figure 1). Primary human CECs (1×10^5 cells/cm^2) were labeled with either Cell Tracker Green or Cell Tracker Red (Molecular Probes, OR), and then plated inside the inserts. For some experiments, CECs plated for 24 hr were pulsed with the manufacturer's recommended dose (Vlahos et al., 1994) of the PI-3 kinase inhibitor LY294002 (50 μM) (Cell Signaling, MA) or DMSO as a control (Sigma-Aldrich, MO) for 30 minutes and washed with serum free media. The Src inhibitor PP2 (Calbiochem) was also used (2 μM) as a specificity control. 72 hr after CECs were plated, the underside of the filters was trypsinized to collect CECs which had either completed or were in the process of completing RPE transmigration

(i.e. those CECs which had migrated across the filter and/or the RPE), and counted with a hemacytometer.

2.3 Nucleofection of CECs

Rac1 inhibition during the transmigration assay was achieved by expression of GFP-POSH-RBD, GFP-17N-Rac1 or GFP alone using the Amaxa basic endothelial cell Nucleofector kit according to the manufacturer's directions (Amaxa Inc.). The construction of GFP-17N Rac1 has been described elsewhere (Wennerberg et al., 2002). The GFP-POSH-RBD was a generous gift from Dr. Antoine Karnoub, University of North Carolina, Chapel Hill, NC and has been described previously (Wennerberg et al., 2002). For expression, 2 μg of DNA per 1×10^6 cells was electroporated prior to plating CECs in the inserts. Expression efficiency was determined by plating an aliquot of the electroporation reaction onto glass coverslips and visualizing GFP fluorescence by microscopy.

2.4 Coculture Assay

2.4.1 Phospho-Akt1 Assay—CECs were grown on 1 μm pore Transwell inserts either solo, in contact with the basal aspects of RPE grown on the underside of the inserts, or in non-contacting coculture with the RPE in the well. After 24 hrs, cells were pulsed with the PI 3-K inhibitor, LY294002 (50 μM) for 30 minutes. Media was replaced and cells were incubated for an additional 20 minutes. RPE were removed from the underside of the inserts using a cotton swab and CECs were lysed in 500 μL of ice cold RIPA buffer plus protease inhibitors and okadaic acid phosphatase inhibitor. Lysates were spun at 4°C for 10 min at 16,000 g. Supernatant total protein was determined using the Bradford method (Pierce, IL) and equalized. 1μg of anti-Akt1 monoclonal antibody (Santa Cruz, CA) was added, and samples were incubated at 4°C overnight with gentle agitation. 30 μL of a 50% solution of protein A-sepharose (Amersham, NJ) was added and samples were incubated 1–2 hr at 4°C with gentle agitation. Beads were washed 3 times with lysis buffer plus inhibitors and resuspended in 30 μL of 2X gel sample buffer (Biorad, CA). Samples were loaded on SDS-PAGE after heating for 10 min at 65°C, and then transferred to PVDF (Millipore, MA). Blots were blocked in 5% BSA/TBST for 1.5 hr at room temperature, then probed for phospho-Akt1 Ser473 (Cell Signaling, MA) or for total Akt1 using the same anti-Akt1 antibody as used for immunoprecipitation above, followed by goat anti-mouse-HRP conjugated secondary antibody (Jackson, PA). Western blots were developed with enhanced chemiluminescence (ECL, Amersham, NJ) and analyzed using Un-scan-it (Silk Scientific, UT).

2.4.2 Rac1 Activity Assay—Cell culture was performed as in the phospho-Akt1 assays above. After RPE were removed, CECs were lysed in 300 μl Rac1 assay buffer B (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$), containing protease inhibitors and 1 mM sodium vanadate. Lysates were clarified by centrifugation at maximum speed (13 000 rpm) for 5 min at 4°C. Total protein concentrations of the lysates were determined using Bradford method (Pierce, IL) and lysates were adjusted to equal protein concentration and volumes, and then incubated for 30 min at 4°C with gentle rocking with 30 μg of GST-PBD (Rac1-binding domain of PAK) fusion protein immobilized on glutathione beads to specifically pulldown active (GTP-bound) Rac1. The beads were washed 4X with the same lysis buffer, then resuspended in 2X gel sample buffer. After SDS-PAGE and transfer of proteins to PVDF membrane, the amount of active Rac1 versus total Rac1 was then determined by Western blotting with a monoclonal anti-Rac1 antibody (Santa Cruz, CA) followed by an HRP-conjugated secondary anti-mouse antibody (Chemicon, CA).

2.4.3 Quantification of Western Blots—Western blots were analyzed by quantitative scanning densitometry using Un-scan-it (Silk Scientific, UT). The relative amount of active Rac1 in each culture condition was determined by measuring the amount of Rac1 sedimented

by the GST-PBD (Active Rac1) relative to the intensity of the bands from the total cell lysates (Total Rac1). Likewise, the relative Akt1 activity was graphically represented by taking the ratio of phospho-Akt1 divided by the amount of total Akt1 in the cell lysates. This method of normalizing relative to the amount of protein in the total cell lysates corrects for any small differences in the loading (Liu and Burridge, 2000).

3. Results

3.1 CEC contact with RPE increases CEC transmigration

We first determined whether "contact" between CECs and the underlying RPE had an effect on transmigration of the CECs across the RPE. We have previously confirmed by confocal microscopy that CECs make direct contact with the RPE plated on the underside of the membrane by extending membrane protrusions through the pores of the Transwell (Geisen et al., 2006a). Because our assay involves CEC migration across not only the Transwell filter towards a chemotactic gradient, as is typical, but also involves migration across the RPE, we extended the time course. We quantified fluorescently labeled transmigrated CECs at 24, 48, and 72 hr after contact with the RPE and compared to transmigrated CECs in solo culture. CEC transmigration was significantly increased by contact with RPE compared to solo culture (Figure 1).

3.2 CEC contact with RPE increases Rac1 activity and involves PI 3-K

Because in many physiological situations cell migration is influenced by the activity of Rho-family GTPases, particularly Rac1, we performed a coculture assay to determine the amount of active Rac1 in CECs grown in contact with RPE compared to CECs grown in coculture without contact, or CECs grown in solo culture (Figure 2A). CECs grown in contact with RPE had significantly higher relative Rac1 activity than those in non-contacting coculture or CECs grown in solo culture (Figure 2B and 2C, (−) LY294002). We found no change in Rac1 activity in RPE (data not shown). To determine if Rac1 activation involved the PI3-K pathway, we selectively treated CECs after 24 hr in culture with LY294002 for 30 min. LY294002 is an inhibitor of PI3-K, which is a signaling protein often found to act upstream of Rac1 (Posern et al., 2000; Kotani et al., 1995). Inhibition of PI 3-K with LY294002 blocked the Rac1 activation we observed during contacting coculture (Figure 2B and 2C, (+) LY294002). Thus, contact with RPE activates Rac1 in CECs through a PI 3-K-dependent mechanism.

3.3 CEC contact with RPE increases Akt1 activation

The involvement of PI 3-K in this pathway was confirmed by using phosphorylation of Akt1/protein kinase Bα as a read-out for PI 3-K activation (Hemmings, 1997; Datta et al., 1999) in response to CEC-RPE contact. Coculture assays were performed as outlined in Figure 2A, and the relative amount of phosphorylated Akt1 was determined by immunoprecipitation and western blot analysis using an antibody specific for active phospho-Ser^{473}-Akt1. As shown in Figure 3A, contacting coculture of CECs with RPE (black bars) increased Akt1 phosphorylation compared with non-contacting coculture (gray bars) or solo culture (white bars). Furthermore, this activation of Akt1 was mediated by PI 3-K, because it could also be blocked by pretreating the CECs with the PI 3-K inhibitor LY294002 (Figure 3).

3.4 Inhibition of PI 3-kinase or Rac1 activity decreases CEC transmigration

We next wanted to determine whether the contact-induced activation of both PI 3-K and Rac1 was required for transmigration of CECs to occur. For transmigration assays (diagrammed in Figure 4A), RPE were plated both on the underside of the Transwell filters and in the bottom of the well to provide soluble chemotactic factors; CECs were then plated for 24 hr, treated for 30 min with either LY294002 or DMSO-only control, and then rinsed. CEC transmigration

was determined 24 hr later. As a control for inhibitor specificity, we also treated CECs for 30 min with 20 μM of the Src inhibitor PP2. Inhibition of PI 3-K with LY294002 significantly decreased CEC transmigration, while inhibition of Src had no significant effect, indicating a specific role for PI 3-K in this phenomenon (Figure 4B). To ensure that the effect of LY294002 at this dose was not due to toxicity, CECs exposed to 0 or 50 μM LY294002 were stained for activated caspase-3. There was no increase in CEC apoptosis even when the incubation time was extended to 24 hrs (data not shown).

Finally, to determine whether Rac1 activation was necessary for CEC transmigration, we inhibited Rac1 activity in CECs two ways. First, we expressed a construct containing the Rac binding domain of the Rac1 effector POSH (GFP-POSH-RBD), which acts by sequestering GTP-bound Rac1 in the cell and thereby preventing it from binding to and activating endogenous Rac1 effectors. Second, we expressed a dominant negative Rac1 construct (GFP-17N Rac1), which acts by binding and sequestering GEFs in an inactive complex. These constructs were introduced into CECs by nucleofection prior to plating, and transmigration was quantified 72 hr later. The assay was extended to 72 hr because at earlier time points the amount of transmigration of all nucleofected cells was low, probably due to a need for the cells to recover after the electroporation procedure. Expression was confirmed by immunofluorescence and western blotting of the GFP tag (GFP only, GFP-POSH-RBD; Supplemental Figure 2); while transfection efficiency was low, there was no significant difference ($p=0.11$) between constructs (GFP: 22.26% +/− 8.94, GFP-POSH-RBD: 27.3% +/− 9.55, GFP-17N Rac1: 38.04% +/− 19.7 percent expressing cells per field). Inhibition of Rac1 activity was also confirmed for those cells expressing GFP-17N Rac1 (Supplemental Figure 3). Because of this relatively low nucleofection efficiency, only green cells (those cells expressing GFP or the GFP-tagged inhibitory constructs) were scored in the transmigration assay. In both cases, when Rac1 activity was inhibited, transmigration of CECs was reduced compared to CECs expressing GFP alone (Figure 5).

4. DISCUSSION

We developed a heterotypic cell coculture model that is relevant to the human disease, AMD. This coculture system allows us to study the effects that contact between CECs and RPE have on signaling components in both cell types; it also recapitulates the event prior to CEC transmigration, an important step in the pathology of neurosensory retina CNV. A good *in vitro* model should retain as much as feasible, the characteristics present in the *in vivo* situation. Our system not only maintains the physiologically relevant orientation of CECs to the basal aspect of RPE, but the RPE develop characteristic polarized distribution of the tight junction protein ZO-1 (Supplemental Figure 1), arguing for a certain state of differentiation. It is interesting to note that a highly differentiated monolayer may not always be relevant to many pathological conditions such as AMD (Geisen et al. 2006b). That said, the degree of differentiation must be kept in mind when interpreting these studies.

Other *in vitro* heterotypic cell culture studies have shown that cell-cell interactions are important for cell proliferation, differentiation and phenotype maintenance. For example, vascular endothelial cells require heterotypic contact with pericytes or smooth muscle cells to maintain vascular phenotype (Sato et al., 200; Sato and Rifkin, 1989) and inhibit proliferation (Saunders and D'Amore, 1992). Furthermore, interactions between endothelial cells and astrocytes are required for induction of blood-neural barrier properties via a mechanism involving release of TGF-β (Garcia et al., 2004). In addition, an *in vitro* coculture model of myoendothelial junctions showed that contact between endothelial cells and vascular smooth muscle cells results in “heterocellular communication” between the two cell types (Isakson and Duling, 2005). With regard to CECs and RPE, previous coculture assays have shown that each cell type is necessary for the mutual survival and phenotypic maintenance of the other

cell type (Sakamoto et al., 1995). However, besides physiologic and beneficial aspects of cell-cell interactions, other studies have pointed to pathologic consequences of heterotypic cell interactions. Coculture of retinal microvascular EC with RPE has been shown to reduce the barrier properties of the RPE in part through the release of VEGF (Hartnett et al., 2003), Other studies have shown the effect of CEC-RPE coculture increasing RPE production of soluble VEGF, which in turn facilitates CEC transmigration (Geisen et al., 2006a). Still, few coculture studies have used a model such as the one reported here to first determine specific proteins activated in either cell type when grown in contact and then to test the effect of their inhibition on function, i.e. on CEC transmigration.

Our coculture model allows us to test a number of possible initiating signals for CEC transmigration: RPE soluble factors such as VEGF, and CEC contact with RPE, and/or RPE-ECM (Figure 6). Interestingly, it appears that there is a cell-type specificity for the induction of CEC transmigration as contact with fibroblast cells or ECM does not promote migration (Supplemental Figure 4). In this study we identified a signaling pathway involving PI 3-K and Rac1 in CECs which stimulates CECs to transmigrate an RPE monolayer (Figure 6). PI 3-K is an important mediator of signal transduction from a variety of cell surface receptors including VEGF receptor 2 (Gille et al., 2000), and one well-known function of PI 3-K is regulation of cell migration. Endothelial cell chemotaxis is dependent upon PI 3-K activation of intracellular signaling cascades (Arefieva et al., 2005) and inhibition of PI 3-K decreases HUVEC migration (Montiel et al., 2005). Similarly, PI 3-K inhibition with LY294002 blocks FGF-2-stimulated corneal endothelial cell migration into scratch wounds *in vitro* (Lee and Kay, 2003), and abrogates the increased CEC migration induced by Beta(3)-adrenergic receptor or NGF stimulation (Steinle and Granger, 2003;Steinle et al., 2005). Our results indicate that CEC transmigration is also PI 3K-dependent and activation of PI 3-K in turn may stimulate intracellular signaling that results in downstream activation of Rac1. While our earlier work showed that CECs increased transmigration in response to a chemotactic gradient of soluble VEGF (Geisen et al., 2006a), the current study identifies another pathway that seems to be independent of any such soluble factor, since non-contacting coculture did not induce activation of Rac1 or PI 3-K. In view of these results, CEC transmigration might be mediated by parallel or synergistic pathways involving both VEGF and contact-induced PI 3-K/Rac1 signaling.

The relationship between PI 3-K and Rac1 during directed cell migration is incompletely understood. PDGF-induced reorganization of the actin cytoskeleton is impaired by inhibition of PI 3-K (Kotani et al., 1994), while constitutively active mutants of PI 3-K stimulate actin filament reorganization in a manner similar to that induced by activated Rac1 (Reif et al., 1996), indicating that there is some interplay. However, there are differing reports as to whether PI 3-K is upstream of Rac1 activation (Hooshmand-Rad et al., 1997; Cheng et al., 2000) or whether it is a downstream effector (Gonzalez et al., 2006). In addition, positive-feedback loops involving Rac1 and PI 3-K apparently exist in some instances (Wang et al., 2002; Srinivasan et al., 2003; Weiner et al., 2002). We found that inhibiting PI 3-K not only blocked CEC transmigration but also inhibited Rac1 activation, suggesting that CEC transmigration is PI 3-K and Rac1 mediated, and that in our system Rac1 is downstream of PI 3-K. Using functional assays, we also confirmed that Rac1 activity is necessary for CEC transmigration by exogenously expressing a dominant negative Rac1 protein (17N Rac1), and by expressing POSH-RBD which sequesters Rac1 effectors.

While our results support a role for PI 3-K and Rac1 during contact-induced CEC transmigration, other possible components in this signaling cascade are still being elucidated. One such possibility is the PI 3-K effector, Akt1/PKB, which is an important cell survival signal and is often used as a downstream reporter for PI 3-K activation (Hemmings, 1997; Datta et al., 1999). However, it is also clear that Akt1 activation itself plays a role in the

regulation of cell migration and VEGF-induced angiogenesis in some situations (Ackah et al., 2005). We found that Akt1 activation coincided with Rac1 activation in our contacting coculture experiments. Does Rac1 and Akt1 activation occur sequentially or in parallel after PI 3-K is activated? Exogenous expression of a constitutively active Rac1 mutant has been shown to increase Akt1 phosphorylation independently of PI 3-K activity (Wennerberg et al., 2002). In addition, sphingosine-1-phosphate activates PI 3-K signaling and Akt1-mediated stimulation of eNOs (Gonzalez et al., 2006), which is abrogated by expression of Rac1 siRNA in these cells. Thus, at least in some systems, Rac1 appears to act upstream of Akt1 activation. Whether or not Rac1 and Akt1 activation is sequentially induced by contact between CECs and RPE in our model system remains to be determined; however, it is clear that both proteins are components of this signaling pathway.

There are yet other components of the signaling cascade initiated by CEC contact with RPE that need to be identified. For example, it is not known what GEFs are acting upstream of Rac1 activation in this system, or what ECM components or cell surface adhesion molecules need to be engaged to mediate “contact” signaling. Furthermore, we have confined our investigation to signaling events which are occurring within the CECs. We know that signaling pathways are also activated within the RPE to release chemotactic factors and cause events such as reduced barrier function (Hartnett et al., 2003), increased CEC chemotaxis (Geisen et al., 2006a), and other adhesion-related events involving integrins (Friedlander et al., 1996) and the extracellular matrix. It is becoming clear that effective treatment of neovascular AMD will likely require several selective treatments targeted at one or more of these specific steps to treat CNV development in the neurosensory retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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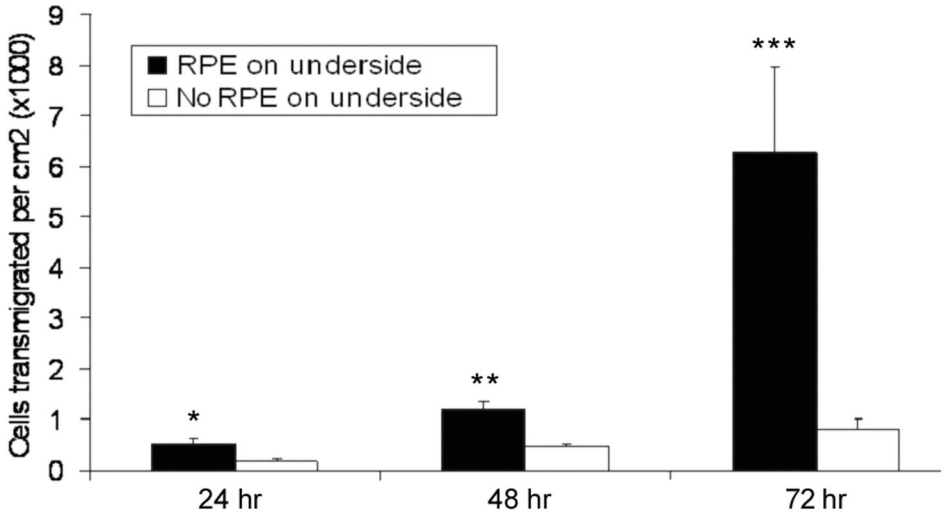

Figure 1. Contact between CECs and underlying RPE increases CEC transmigration

Transmigration of CECs was quantified after 24, 48, or 72 hr in coculture or solo culture. There is a significant increase in CEC transmigration when CECs are cultured in contact with RPE versus solo CEC culture. Data represent the mean of 3 experiments +/−SEM (n=3) *p=0.012, **p=0.004, ***p=0.016, Student's *t*-Test.

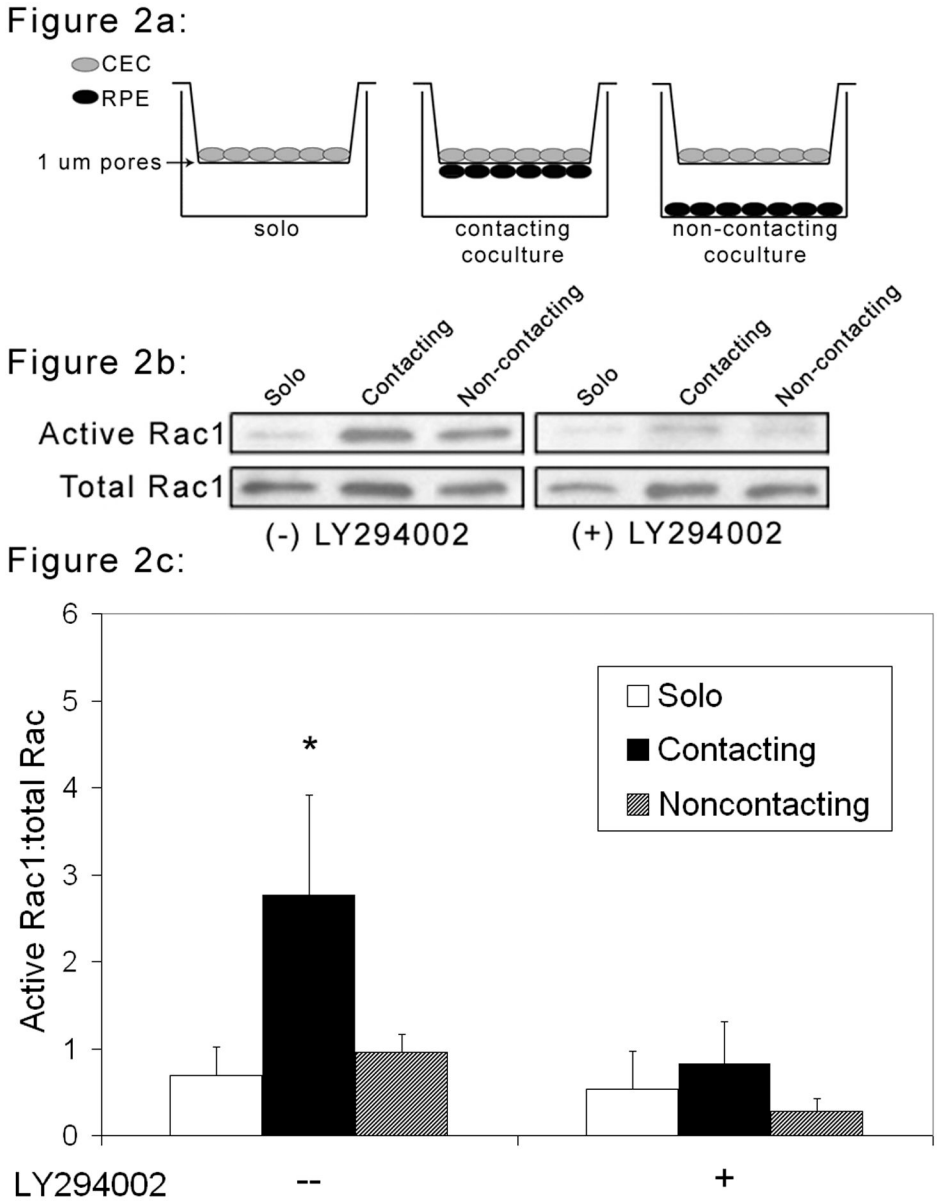

Figure 2. Contact between CECs and underlying RPE increases Rac1 activity; an effect which is dependent on PI 3-K activity

(A) Schematic representation of coculture assay used for biochemical analysis in CECs. Solo culture indicates CECs are plated on the upper surface of the Transwell insert, with no RPE present. For contacting coculture assays, CECs are grown in a Transwell insert with small (1 μm) pores that permit CEC processes to contact the basal aspect of an RPE monolayer grown on the underside of the Transwell insert. In non-contacting coculture, RPE are grown in the lower well with CECs in the insert. (B) CECs grown in solo, contacting, or non-contacting coculture for 24 hr were pulsed for 30 min with LY294002 (50 μM) or DMSO vehicle control, rinsed, then assayed for Rac1 activity 20 min later. The levels of active Rac1 increase with contacting coculture, and this increase is blocked by LY294002 pretreatment. A representative blot of active Rac1 sedimented by GST-PBD versus total Rac1 from whole cell lysates is shown. (C) Quantification of blots: average relative Rac1 activity (+/− SEM) is obtained by

averaging the ratio of active to total Rac1 from 4 independent experiments. Overall ANOVA, $p=0.0217$, post-hoc Student-Newman-Keuls Multiple Comparison showed significant differences between contacting CECs (*) and solo $p<0.05$) or non-contacting CECs ($p<0.05$) in the absence of LY294002.

Figure 3a:

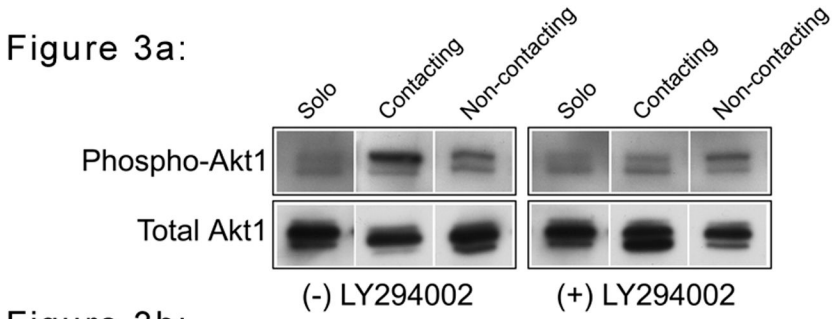

Figure 3b:

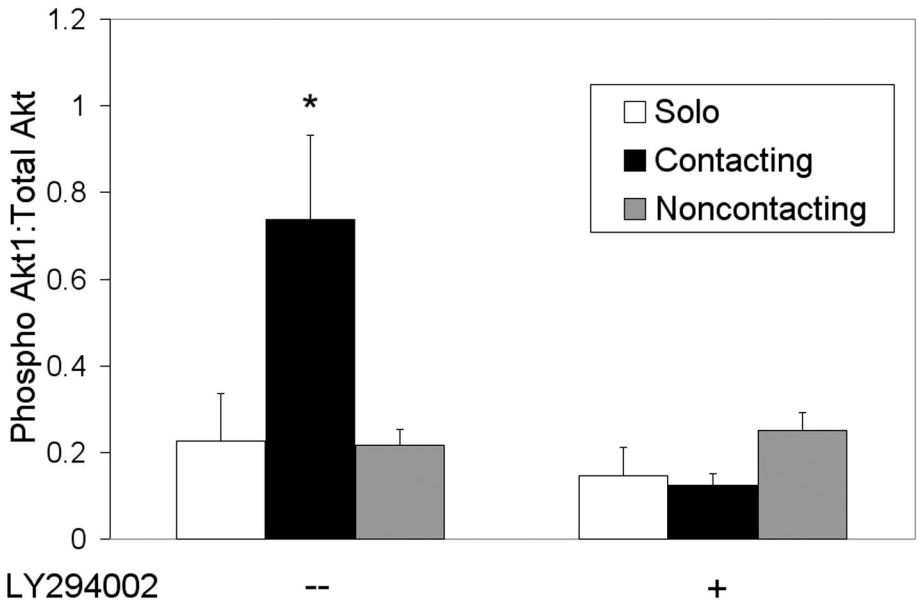

Figure 3. Contact between CECs and underlying RPE activates Akt1 via PI 3-K

(A) CECs in solo, contacting, or non-contacting coculture (as in Figure 2A) were grown for 24 hr and assayed for activation of PI3K/Akt1. Akt1 immunoprecipitates were analyzed for either total Akt1 levels or active Akt1, using a phospho-specific antibody. Contact between CECs and RPE increased Akt1 phosphorylation, and this increase was blocked when CECs were pulse-treated with the PI 3-K inhibitor LY294002 as described (30 min, 50 μM). A typical blot of total versus active (phosphorylated) Akt1 is shown. (B) Graph represents the average relative phosphorylation of Akt1 (+/− SEM) from 3 independent experiments. Overall ANOVA, $p=0.0039$, post-hoc Student-Newman-Keuls Multiple Comparison tests showed significant differences between contacting CECs and solo ($p<0.01$) or non-contacting CECs ($p<0.001$) in the absence of LY294002.

Figure 4A:

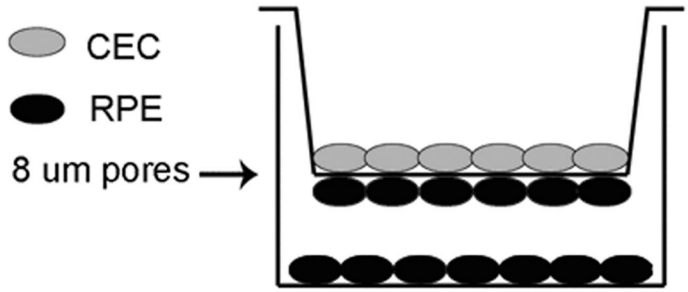

Figure 4B:

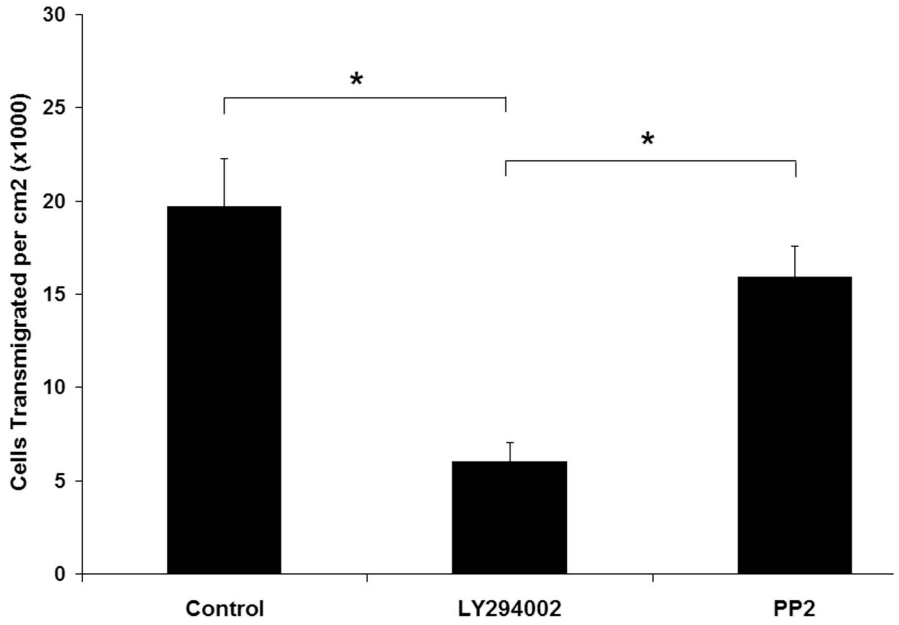

Figure 4. Inhibition of PI 3-K reduces CEC transmigration

(A) Schematic representation of transmigration assays. CECs and RPE are grown in coculture on either side of a Transwell insert with large enough pores (8 μm) to permit transmigration. RPE are plated in the lower well to provide chemoattractants. (B) CECs grown 24 hr in transmigration assays were pulsed for 30 min with 50 μM LY294002, 2 μM PP2, or DMSO-only control. After 24 hr, transmigrated CECs were quantified. Transmigration was inhibited specifically when PI 3-K/Akt1 activity was blocked. Graph represents mean +/− SEM of 3 independent experiments. *$p < 0.01$

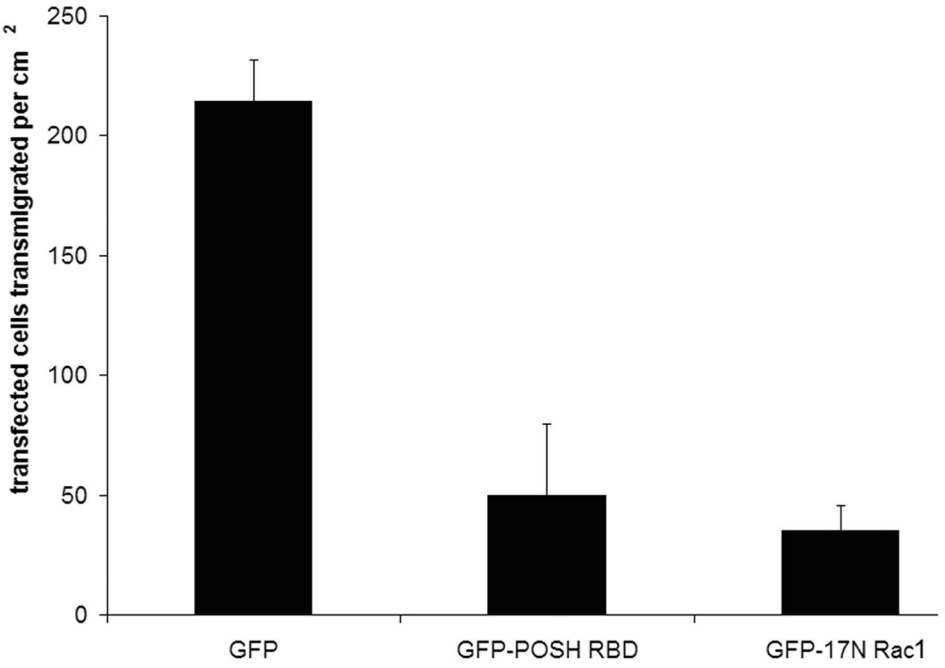

Figure 5. Inhibition of Rac1 activation reduces CEC transmigration

CECs were nucleofected to express either GFP alone, or GFP-POSH-RBD and GFP-17N Rac1 to inhibit Rac1 activity, and then grown in contacting co-cullture with RPE. After 72 hr, transmigration of CECs expressing these constructs was quantified by counting only green cells from the underside of the filter. Inhibition of Rac1 by expressing both GFP-POSH-RBD and GFP-17N Rac1 significantly reduced transmigration compared to GFP-expressing cells. Graph represents mean +/− SEM of 4 independent experiments with GFP-POSH-RBD and 3 experiments with GFP-17N Rac1. $p<0.001$ GFP compared to GFP-POS-RBD or GFP-17N Rac1.

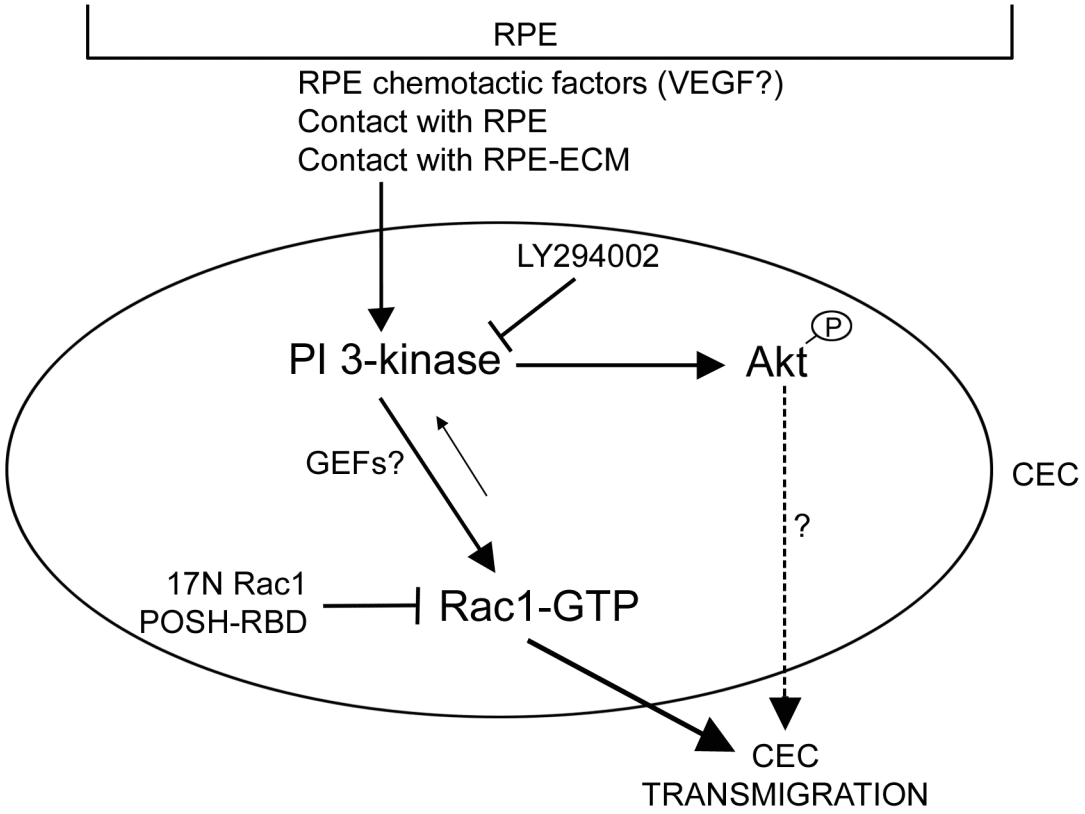

Figure 6. A Rac1 and PI 3-K-dependent signaling pathway is central to the contact-induced migration of CECs

Putative signals which stimulate CECs to migrate include: chemotactic factors such as soluble VEGF secreted by RPE, contact between CECs and RPE, or contact with RPE extracellular matrix. Rac1 GTPase is activated via a PI 3-K-dependent mechanism in response to any or all of these stimuli, with the end result being transmigration of CECs across the RPE.